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EXOTOXINS[1]

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The most interesting aspects of bacterial exotoxins are the physiology of their production, their mode of action, and the part they play in disease. Therefore, it is embarrassing to record that little is known of any of these subjects, despite the large number of papers on exotoxins (more than 1500) that have been published in the last ten years. The physiology of production of not a single toxin is understood (although some progress has recently been made with diphtheria toxin in this connection). The mode of action of only one classical bacterial toxin can be defined in chemical terms insofar as it is possible to define the group of substances it acts upon and the results of this action. This is the alpha-toxin of *Clostridium perfringens* which was recognized as a phospholipid-splitting enzyme by Macfarlane & Knight more than twenty years ago (89). The pathology associated with only three toxins, viz., those of diphtheria, tetanus, and botulism, is properly understood, and this understanding is not new since it dates from the last century.

The recent finding that two bacterial toxins release degradative enzymes from lysosomes [see (33)] may have wider applications and may be important in understanding the immediate consequences of the fundamental action of some toxins, if not the fundamental action itself. Weissman, Keiser & Bernheimer [(169); see also (60)] showed that the hemolytic toxins, streptolysins O and S, liberated β-glucuronidase and acid phosphatase from the large granule fraction of rabbit liver, heart, spleen, and lymph nodes. Perhaps other hemolytic (and nonhemolytic) toxins may prove to be capable of liberating degradative enzymes (e.g., cathepsin, DNase, RNase, phosphatase, β-glucuronidase) from the lysosomes in the tissues of the host. These degradative

[1] The survey of the literature pertaining to this review was concluded in November 1963.

enzymes might then be responsible for the harmful, often shocklike, effects of some bacterial toxins. In clinical gas gangrene, toxin-producing organisms grow and produce toxin in the tissues of the host and the host is in a state of shock, but no gas gangrene toxin is detectable systemically and systemic specific antitoxin is ineffective. Yet amputation of the infected limb brings relief and apparently removes the source of shock-producing substances [see (90)]. It is conceivable that these shock-producing substances may be degradative enzymes (or may arise from the action of such enzymes) from the lysosomes in the tissues of the host.

A number of bacterial toxins have proved to be complex. Diphtheria toxin, which contains at least four antigenic determinants, or is a mixture or complex of four antigens, and the staphylococcal leucocidin, which consists of two synergistically active proteins, are discussed in some detail below. Anthrax toxin contains three synergistically active protein components. Factor I is not toxic when injected alone but together with factor II it evokes edema in the skin of the rabbit and kills mice. The mixture of factors I and II is less lethal per unit of edema-producing activity than the crude toxin, and this led to the demonstration of a third factor. Factor III differed serologically from factors I and II, was nonlethal by itself or when mixed with factor I, but was lethal when mixed with factor II, and increased the lethality of mixtures of factors I and II per unit of edema-producing activity (55, 133, 137–142, 150). The toxicity of the plague bacillus appears to be due to a mixture of two components which are unequally divided between the material extractable from the cell and the cell wall material (30, 136).

The numerous toxins and enzymes of the histotoxic anaerobic clostridia were recently reviewed in detail by MacLennan (90).

Diphtheria Toxin

The physiology of diphtheria toxin production.—There are virulent and avirulent, and toxinogenic and nontoxinogenic strains of *Corynebacterium diphtheriae*. Virulence and toxinogeny are not the same. Virulence, the property of becoming established and growing on the mucous membrane or in the skin, is under genetic control of the bacterium (10), but the gene governing toxinogeny resides in certain bacteriophages. A nontoxinogenic strain of the diphtheria bacillus infected with a temperate phage is converted to a stable state of lysogeny and toxinogeny [Barksdale (8); Barksdale & Pappenheimer (12); Freeman (39, 40); Groman (50, 51, 52)]. All toxinogenic strains of the bacillus are lysogenic, i.e., they carry a prophage, but not all lysogenic strains are toxinogenic, because not all corynebacterial phages are toxinogenic [Groman (51)], and because some bacilli which become lysogenized by a toxinogenic phage do not become toxinogenic [Barksdale (9)]. Reversion to nonlysogeny always means reversion to nontoxinogeny [Groman (51)]. When toxinogenic and nontoxinogenic phages which have different host ranges are crossed, a nontoxinogenic recombinant phage may be isolated with the same host range as the toxinogenic phage [Groman & Eaton (53)].

Barksdale believes that toxin production takes place in diphtheria bacilli that are lysing as a result of phage multiplication (8, 10, 11). It is well known that an excess of iron suppresses toxin production. It is also known that toxin is not secreted by or contained in logarithmically growing cultures (104, 126), but is released into the culture medium during the phase when growth is declining as a result of the depletion of iron (97). Barksdale suggested that the depletion of iron leads to a depletion of catalase which, in turn, leads to an accumulation of peroxides that bring about "autoinduction" and lysis. When lysogenic toxinogenic cells were induced to lyse by irradiation with ultraviolet light, phage was liberated, and when the burst was complete, toxin was also released linearly with time, but only if the iron content of the suspending medium was very low. If iron was added during the release of toxin, further release was blocked at that stage. From these and other experiments Barksdale concluded that in the diphtheria bacillus prophage acts as a gene governing toxinogeny because it acts as a built-in source of phage, the multiplication of which is necessary for toxin production.

But Yoneda & Pappenheimer do not believe that an important proportion of the toxin is liberated as a result of phage multiplication and lysis of the bacterial cell (109, 185). Like Barksdale, they found that when cultures of the diphtheria bacillus were suspended in iron-free medium the organisms immediately went into a phase of declining growth and some free phage and some toxin was liberated. But the degree of lysis accounted for the liberation of only 1 μg protein/ml, whereas 12 μg toxin/ml were liberated. They found also that when diphtheria bacilli labelled with radioactive methionine were transferred to an iron-free, nonradioactive medium, growth increased from about 3 to about 6 mg dry weight/ml, and extracellular toxin from 0 to 35 Lf/ml, but the radioactivity per milliliter of culture filtrate remained constant, i.e., that of the toxin decreased. The small residual radioactivity of the toxin was probably carried over from the inoculum. With radioactive phenylalanine-labelled cells they found similar results and concluded that the toxin was synthesized *de novo* from the amino acids in the fresh, iron-free nonradioactive medium. They found that while toxin was being produced during the declining phase of growth in iron-free medium, the growth mass increased four- to fivefold as measured by estimations of optical density, dry weight, and intracellular fumarase. Nucleic acids, the presence of which in the culture fluid signifies the breakdown of bacterial cells, appeared in the culture fluid only after toxin production and growth had ceased. They concluded that whether or not the increased bacterial mass was due to actual cell division of bacteria, the degree of lysis of the culture was too insignificant to account for the release of toxin.

Interesting studies have been made of the protein produced by nontoxinogenic strains of the diphtheria bacillus under conditions in which toxinogenic strains produce toxin. When cultures of nonlysogenic and lysogenic strains (C7 and C7 (β)) were transferred to an iron-free medium they entered into the declining phase of growth and measurable amounts of por-

phyrin and protein were secreted within 1 to 2 hr. Both strains produced the same amount of protein and porphyrin, but in the case of the lysogenic strain 50 percent or more of this protein was toxic [Yoneda, Ishihara & Okuda (184); Yoneda & Pappenheimer (185)]. About 40 per cent of the protein was precipitable between ⅓ and ⅔ saturation with ammonium sulphate. In the case of the lysogenic strain, this fraction of protein was toxic, antigenic, and largely monodisperse; in the case of the nonlysogenic strain it was nontoxic, nonantigenic, and polydisperse. Yoneda & Pappenheimer (185) suggested that the nonlysogenic strain contained a factor, perhaps a protease, that broke down the toxin into a number of nontoxic, nonantigenic fragments. Mixing a nonlysogenic culture with a lysogenic culture in an iron-free medium did not affect the toxin produced by the lysogenic culture, which suggested that a destructive factor was not liberated extracellularly by the nonlysogenic strain. It seems more likely that if such a factor existed it would be intracellular rather than extracellular, but it does not appear to have been sought intracellularly.

Some years ago, Pappenheimer (104, 106, 111, 112) suggested that diphtheria toxin might be closely related to the protein moiety of the bacterial cytochrome-*b*. In the presence of iron the organism produces cytochrome-*b*; in the absence of iron it continues to produce the protein and porphyrin moieties of cytochrome-*b* but is unable to complete the synthesis of the respiratory pigment. The protein and the porphyrin are therefore secreted and the protein is the toxin, or closely related to it. This ingenious hypothesis has not yet been proved, but nothing that is known about the relationship of phage to toxin is inconsistent with it. Some of the polydisperse protein produced by nonlysogenic strains of the diphtheria bacillus in the absence of iron may also be related to the protein moiety of cytochrome-*b*. Lysogeny and toxinogeny also appear to be related in the staphylococcus (18), the streptococcus (187), and *Bacillus cereus* (1).

The nature of diphtheria toxin.—Our immediate predecessor (101) concluded his review of bacterial toxins with a critical discussion of the purity of the preparations of diphtheria toxin that had been made by Eaton (37) and by Pappenheimer (103) nearly twenty years previously. This discussion, which involves also a consideration of the now sterile subject of the virtues and faults of quantitative toxin-antitoxin flocculation, has been continued by Pappenheimer & Yoneda (113), Pope (115) and Marrack (95). We do not propose to cover this ground again, but will suggest that the parties on both sides of this stimulating and fruitful debate are essentially correct in the views they maintain. It seems to us that the prewar preparation of Pappenheimer does not differ in an important way from the postwar preparations that have been obtained in England [Pope & Stevens (117, 118)]; the United States [Lepow & Pillemer (83)]; Sweden [Norlin (100)]; France [Raynaud & Relyveld (125)]; and Japan [Katsura et al. (72)].

Pappenheimer's (103) preparation undoubtedly was inhomogeneous. Although apparently homogeneous by contemporary electrophoretic and

ultracentrifugal criteria (88, 108), it contained at least 14 antigens as revealed by gel-diffusion against antitoxin [Pope et al. (123)], and only 2170 Lf/mgN, compared with the single immunodiffusion line and the value of 3200 Lf/mgN in Pope & Steven's (117) crystalline preparation. On dilution, however, most of the diffusion lines disappeared and it is evident that the extraneous antigens did not amount to a considerable proportion of the whole [Bowen (24)]. On the basis of the published values of Lf/mgN it would appear that Pappenheimer's preparation contained about 70 per cent as much toxin (plus toxoid) as the best preparations made so far, but private communication with the principals concerned suggests that this matter cannot be settled without a further exchange of samples of toxin and antitoxin. Like Marrack (95), we regard material of about 3000 Lf/mgN as essentially pure diphtheria toxin. It is consistently isolated in many laboratories and behaves as a single kinetic and immunological unit. Eaton and Pappenheimer deserve credit for having made preparations not seriously inferior to this more than twenty-five years ago, when no other bacterial protein had been obtained in a comparable state of purity.

But this is not to say that diphtheria toxin is necessarily a simple single protein. Pope & Stevens (118–122) have shown that the purified toxin either has several antigenic determinants or is a complex, or a consistent mixture, of several antigens, of which possibly only one is toxic. The crystalline toxin preparation of Pope & Stevens showed only one line on immunodiffusion against antisera from horses hyperimmunized against crude toxin; conversely, sera from horses hyperimmunized against the crystalline toxin gave only one line against crude toxin. But if the crystalline toxin was partially denatured by heating or by treatment with alkali, the single diffusion line would separate into four lines; if the crystalline toxin was partially digested with pepsin or trypsin, three lines were obtained. The new separated lines all showed reactions of identity with the original single line Similar results have been obtained with human and bovine serum albumin by Lapresle (78, 79, 80, 167) and Weigle (168). In these cases the multiple diffusion lines were ascribed to multiple antigenic determinants in a single protein molecule reacting with separate specific antibodies; however, Pope suggests that diphtheria toxin is not a single protein with several antigenic determinants, but a mixture of several distinct antigens.

Two of the antigens (or antigenic fragments), the "phosphate-stable" antigen and a "pepsin-stable" antigen [there is a suggestion that there are two pepsin-stable antigens (122)], were obtained in immunologically reasonably pure states. The "phosphate-stable" antigen was, in fact, an alkali-stable antigen and could be obtained by treatment of the crystalline toxin with NaOH at pH 11–12. It was more conveniently obtained by treating the toxin with 0.5 per cent Na_2SO_3+0.25 percent K_3PO_4 at pH 11 at 35° C. This treatment destroyed the other antigens. The "pepsin-stable" antigen was obtained by treating the crystalline toxin with pepsin at pH 3.7 and separating it from the digestion products by salting out with ammonium sulphate.

On adding increasing concentrations of the untreated crystalline toxin to fixed concentrations of antitoxin, a flocculation curve was obtained with a slanting, nearly linear top, similar to that first observed by Pappenheimer & Robinson (110). The phosphate-stable and pepsin-stable antigens give parabolic flocculation curves. Pope & Stevens believe that the slanting top of the curve with untreated toxin is due to the overlap of the parabolic curves of the component antigens. The phosphate- and pepsin-stable antigens appeared to account for about 65 per cent of the flocculation obtained with the untreated crystalline toxin. Neither of these antigens was toxic. When either of these reacted with antitoxin, the supernate of the floccules failed to give an immunodiffusion line with the antigen in question but did give a line with the other antigen. In both cases the supernate retained more than 80 per cent of antibody capable of neutralizing the toxicity of the untreated toxin. Similar results were obtained by Fulthorpe (42).

Ambrose & Easty (5) examined the single immunodiffusion line of the crystalline toxin by an interferometric method and observed that it was composed of at least four closely packed very sharp lines. Pope & Stevens (119) observed that although a single line was seen on immunodiffusion of the crystalline toxin against most antitoxic sera, it was not seen with all antitoxic sera. Five of 37 antitoxic sera gave two lines.

All of the observations so far discussed could be accounted for if the toxin were a single protein with several antigenic determinants, each stimulating the formation of a separate antibody. But Pope believes that the crystalline toxin contains several independent antigens. The observations of Lapresle and Weigle (see above) on the antigenic determinants of albumin were made after the albumin had been partially digested with proteolytic enzymes and under these circumstances a cleavage of peptide bonds in a single protein molecule is to be expected. But in addition to proteolytic attack on the crystalline diphtheria toxin, Pope also obtained separation of diffusion lines under circumstances (treatment with sulphite, urea, phosphate, heating) in which a hydrolytic cleavage of peptide bonds was unlikely. The possibility of independent molecules held together by weaker forces than covalent bonds (cf. crystalline protamin insulin), seems more likely. The suggestion that the crystalline diphtheria toxin may not be a singular molecular species has further support. In addition to the four antigens (or antigenic determinants) which are always present in crystalline immunologically and kinetically homogeneous toxin preparations, there may be other components which are sometimes but not always present and therefore unessential to the toxin. Crystalline toxin preparations derived from cultures grown on tryptic digest media contained a deoxyribonuclease, toxin derived from papain digest media did not [Pope (115)]; some crystalline toxin preparations contained a trypsin-stable antigen and others did not [Pope & Stevens (121)]. The deoxyribonuclease was neutralized and precipitated by antisera from horses immunized with toxoid prepared from tryptic digest culture filtrates but not by antisera from horses immunized with toxoid derived from papain digest fil-

trates. If the idea of an independent deoxyribonuclease molecule, or of a trypsin-stable component, in the crystalline toxin preparation is considered, then other independent molecules (as distinct from antigenic determinants) must also be considered. Molecular inhomogeneity in crystalline immunologically and kinetically homogeneous toxin preparations is also suggested by Pope's (116) observation that when toxin was adsorbed on DEAE cellulose a number of fractions differing in chemical composition and biological activity could be eluted.

Pope & Stevens appear to have established a good case for the idea that the crystalline toxin, although immunologically and kinetically homogeneous, is not chemically homogeneous. It is likely that it is a mixture or complex of several molecular species rather than a single molecular species with several antigenic determinants. This preparation has been consistently isolated in many laboratories all over the world, and even if it is a mixture of molecular species it has a consistency which must be significant in the physiology of the diphtheria bacillus. The whole complex is diphtheria toxin. It is possible that the toxicity of the complex is due to one of its components, but all attempts to isolate such a toxic component have failed, and there is no evidence that the toxicity is not dependent on the integrity of the complex as a whole. It is difficult to conceive of the components of the complex not being bound together in some way; otherwise the apparently single immunodiffusion line (resolvable only interferometric methods) would require an astonishingly consistent and integrated balance of the concentrations, diffusion coefficients, and optimal precipitation ratios of the several separate antigens and their homologous antibodies.

The mode of action of diphtheria toxin.—Diphtheria toxin produces an area of necrosis when injected into the skin. It is toxic to all tissues of the body of the susceptible animal and its toxicity to tissues grown in culture was demonstrated 50 years ago by Levaditi & Mutermilch (84). New methods of tissue culture have made possible the detailed investigation of the action of the toxin on mammalian cells. Placido Souza & Evans (114) and Lennox & Kaplan (82) showed that cultures of human, monkey and rabbit kidney cells, human amnion cells, human epithelial carcinoma cells, and HeLa cells could be used in titrating the toxin and its antitoxin. From the data of Lennox & Kaplan (82) on rabbit kidney cells and of Gabliks & Solotorovsky (43) on human kidney cells, it can be calculated that 200 to 400 molecules of toxin will kill one cell.

The cytopathogenic effects of diphtheria toxin on tissue cultures appear long after profound chemical changes have taken place. Strauss & Hendee (144) observed that incorporation of radioactive methionine was completely inhibited within about 90 min of the addition of toxin to a culture of HeLa cells, whereas cytopathogenic effects first became visible in a few cells after 4 hr. Methionine incorporation (i.e., protein synthesis) was blocked by a concentration of toxin as low as 0.01 Lf/ml and saturation (maximum effect at) was reached at 0.2 Lf/ml (0.5 μg/ml). There was no effect on the aerobic

respiration of the HeLa cells and glycolysis fell off only after some hours, when cytopathogenic effects became visible.

During the period of normal growth and protein synthesis, the toxin was neutralizable by antitoxin, even if added 30 min after the toxin. Pappenheimer & Collier (105) showed, with tritium-labelled toxin, that probably less than 1 per cent of a saturating dose of toxin was fixed or taken up by the cells in the first 5 hr. They concluded that only a few molecules of toxin acted per cell, even in the presence of high toxin concentrations, and suggested that the toxin acted catalytically within the cell. The action of the toxin is probably not on the cell membrane since Kato & Pappenheimer (71) showed that the uptake of K^{42} by monkey kidney cells was normal for several hours in the presence of a saturating dose of toxin, and Strauss & Hendee (144) showed that there was no leakage of P^{32} from intoxicated cells. Kato & Pappenheimer (71) confirmed the blockage by toxin of incorporation of radioactive methionine. Strauss (143) showed that the block in protein synthesis was not due to an effect on the level of ATP in the cells, but Kato & Pappenheimer (71) observed a 40 per cent decrease in the steady-state level of incorporation of inorganic phosphate into ATP in intoxicated human kidney cells. However, Pappenheimer & Collier (105) were subsequently unable to confirm this finding and obtained convincing evidence that the cessation of protein synthesis in intoxicated HeLa cells could not be ascribed to an effect of the toxin on intracellular levels of either ATP or GTP.

The same investigators (105) have studied the effect of diptheria toxin on the synthesis of protein by a cell-free system extracted from HeLa cells ruptured by means of a French pressure cell. The extract obtained after centrifuging down the ruptured cells readily incorporated radioactive amino acids into the trichloracetic acid-precipitable material when external ATP and an ATP-generating system were provided. It was highly sensitive to puromycin (a drug which immediately inhibits protein synthesis in cell cultures) and ribonuclease, but not to deoxyribonuclease. With such an extract, toxin at a concentration of 0.5 μg/ml rapidly caused a 50 per cent inhibition of incorporation of C^{14}-leucine. Kato (70) also obtained an inhibition of incorporation of labelled amino acids in the proteins of a cell-free system from guinea pig liver, but with a concentration of diphtheria toxin a thousand times as high, and the possibility remains that the effect was due to contaminants of the toxin. Such contaminants might be ribonuclease or deoxyribonuclease, the former known to be a potent inhibitor of protein synthesis. Pappenheimer & Collier (105) ruled out ribonuclease by showing that their toxin preparation did not inhibit C^{14}-amino acid incorporation in a cell-free system from *E. coli* that was 100 times more sensitive to pancreatic ribonuclease than the HeLa system, and by showing that their toxin preparation did not break down the synthetic ribonucleotide, polyuridilic acid. They also showed that high concentrations of deoxyribonuclease did not inhibit amino acid incorporation in the HeLa system.

Pappenheimer & Collier (105) made the interesting observation that

although antitoxin inhibits the effect of toxin on intact HeLa cells (and, of course, the whole animal), toxin-antitoxin floccules retained a significant toxicity against cell-free extracts. It is well known that the action of many enzymes on their substrates is not inhibited by homologous antibody and it is not unlikely that the inhibitory effects of at least some antitoxins may be due to the inability of toxin-antitoxin complexes to penetrate into cells. [Anti-urease inhibits urease only 20 per cent *in vitro* but completely protects rabbits against the lethal effects of the enzyme; for further discussion [see van Heyningen (153).]

Pappenheimer & Collier (105) have made some progress in finding the step in protein synthesis that is blocked by diphtheria toxin. During protein synthesis each amino acid reacts with specific soluble RNA (sRNA) in the presence of a specific activating enzyme and ATP to yield aminoacyl-sRNA and adenylic acid. The various species of aminoacyl-sRNA then interact with messenger RNA (which is attached to ribosomes) and transfer their amino acids to growing polypeptide chains. This step needs transfer enzymes, GTP, K^+ and Mg^+. They showed that in the HeLa cell-free system toxin blocked the incorporation of C^{14}-phenylalanine into polyphenylalanine that was stimulated by the artificial messenger RNA, polyuridilic acid. When a similar effect of the toxin was sought in a partially purified cell-free system from rabbit reticulocytes it was not found, although this system incorporated amino acids to a much greater extent than the HeLa system. However, blockage by toxin of polyphenylalanine synthesis by the reticulocyte system was observed when a supernate of the HeLa extract containing enzymes, sRNA, and cofactors was added to it. This supernate was obtained by centrifuging out the microsomal fraction of the HeLa extract at 105,000 $\times g$. for 90 min. Evidently this supernate contained a factor which, although not necessary for protein synthesis by the reticulocyte preparation, was necessary for blockage of protein synthesis by the toxin. The nature of this factor has not yet been determined but it appears to be a small molecule since the sensitivity to toxin of the reticulocyte system + HeLa supernate was reduced when the HeLa supernate was dialyzed.

Staphylococcal Toxins

The first step in understanding the mode of action of the many active products of the staphylococcus [see (15)], and the part they play in disease, must be their isolation in pure form. Attempts have been made to purify a number of these substances. Beside the alpha toxin, the Panton-Valentine leucocidin, and the enterotoxin which will be discussed in greater detail, purification of the following products has been achieved with varying degrees of success: beta toxin (2, 51, 67), delta toxin (68, 186), coagulase (19, 36, 65, 132), staphylokinase (48), and nuclease (99, 124). Phospholipase activity has been detected in staphylococcal filtrates (34, 93, 127, 128, 134).

Staphylococcal alpha toxin.—Numerous attempts have been made to purify the staphylococcal alpha toxin (29, 49, 66, 76, 77, 87, 92, 130, 152, 172), the

most successful being that by Bernheimer & Schwartz (16). Their method of purification involved ammonium sulphate precipitation and fractionation of the crude toxin from the culture filtrate, followed by curtain electrophoresis, zone electrophoresis in a density gradient, and ultracentrifugation in a density gradient. As by-products of the purification process, two proteins were obtained in crystalline form. Their biological activity has not yet been determined. The purified toxin preparation was not completely homogeneous and could be resolved by electrophoresis in a density gradient into four components that did not differ strikingly in biological properties. Two of these components were considerably less hemolytic per unit weight than the others, but their hemolytic activity could be increased eighteen-fold by dialysis against ethylenediaminetetracetic acid, which suggests that they consisted largely of inactive toxin capable of activation. The most striking feature of the amino acid analysis of the toxin was the complete absence of cystine. The average molecular weight was 44,000. A liter of culture filtrate may contain as much as 84 mg of alpha toxin, i.e., 2 per cent of the dry weight of the cocci that produced it.

Nothing is known of the mode of action of the alpha toxin. The numerous studies that have been made with smooth and striated muscle (6, 86, 148, 171), tissues (7, 81, 91, 147), and red blood cells (85, 94, 170) have so far not thrown any light on this subject. It has been suggested that the toxin may be a proteolytic enzyme (130), but the purified preparation of Bernheimer & Schwartz had no proteolytic activity (16).

Staphylococcal leucocidin.—There are at least three distinct staphylococcal leucocidins: the alpha hemolysin, active only on rabbit leucocytes without grossly changing their morphology; the delta hemolysin, active on leucocytes of all species, and lytic as well as lethal; the nonhemolytic Panton-Valentine leucocidin, i.e., the "true" leucocidin active on rabbit and human leucocytes with characteristic changes in morphology. Gladstone & van Heyningen (47) observed that when the Panton-Valentine leucocidin attacked human polymorphonuclear leucocytes, changes took place within a few minutes. First there was a loss of motility of the cell and withdrawal of pseudopodia. The cytoplasmic granules lost their orderly streaming and underwent Brownian movement. After a few minutes the cells became spherical but not greatly swollen, and most of the granules disappeared. The remaining granules lost their Brownian movement and became closely applied to the cell wall. There was no disruption of the cell. Leucocidin-treated leucocytes lose their ability to reduce the dyes methylene blue (98) and phenol-indo-2:6-dichlorphenol in the presence of cyanide, and this reaction can be used in the assay of the leucocidin. Macrophages are preferable in the assay because they last longer *in vitro* (69, 174).

Work on the nature of the Panton-Valentine leucocidin and the biochemical changes it brings about in the leucocyte was then taken up by Woodin, and reported in a series of interesting papers which will now be discussed in a

different order from that in which they appeared. Leucocidin-treated leucocytes were observed by light- and electron microscopy (179). When calcium was present in the suspension medium of the treated cells they lost their granules, and vesicles could be seen in the cytoplasm and in the medium. Some of the vesicles were fused to the surface of the cell. In the absence of calcium, the Brownian movement of the granules persisted for at least 30 min and there was no degranulation and no vesicles were formed. When calcium was added to leucocidin-treated cells after incubation in calcium-free medium, the Brownian movement stopped, some granules disappeared, and vesicles could be seen in the cytoplasm. Measurements of specific activities of some enzymes (acid phosphatase, β-glucuronidase, peroxidase; see below) in the granules and vesicles suggested that the vesicles were produced from the granules, some of which had discharged part of their contents. The conversion of granules to vesicles may have been simultaneous with the fusion of the granules to the cell surface. It was suggested that the vesicles released their contents to the exterior of the cell following rupture or change of permeability of the cell membrane at the site of fusion only (179).

The granule contents which were thus selectively extruded by the leucocidin-treated cell consisted of protein. Woodin had shown that protein was released from treated leucocytes; it was not derived from the soluble protein of the cell, and probably not from cell surface since the change in the permeability of the surface was small and the amount of protein released relatively large (176). It appeared to be derived from the granules because many of the enzymes of the granules (lysosomes?), viz., lysozyme, phagocytin, ribonuclease, deoxyribonuclease, β-glucuronidase and peroxidase could be demonstrated in the extruded protein of the leucocidin-treated cells. The increased amounts of these enzymes in the supernate of the treated cells corresponded with their decrease in the granule fraction (178). The extrusion of protein from the leucocidin-treated cells did not take place unless calcium was present in the suspending medium (181). When calcium was present it accumulated in the leucocidin-treated cells and at the same time there was a loss of magnesium. When calcium was added to leucocidin-treated cells suspended in calcium-free medium, the extrusion of protein was induced. Leucocidin did not play a direct part in the extrusion of the granule contents because the addition of calcium to the leucocidin-treated leucocytes after neutralization of the leucocidin with antibody still induced the release of protein. The calcium appeared to play a part in the adherence of the granules to the cell wall, since Brownian movement of the granules continued for a long time when leucocidin-treated cells were suspended in a calcium-free medium, and stopped when calcium was added, even at 0° C.

Woodin points out that there have been speculations (129) that leucocytes may have a secretory function, but that so far there has been no direct demonstration of the selective transfer of granule proteins to the exterior of the cell, except in the case of the leucocidin-treated leucocyte. An analogous

phenomenon in which fusion of the granules with the cell surface occurs takes place after phagocytosis (30, 59). It is probable that the normal leucocyte possesses the equipment for extruding the granules contents, and all that leucocidin does is to change the geography of the cell. The mode of extrusion of protein from the leucocidin-treated leucocyte is analogous to that of normal mammalian cells, e.g., the acinar cells of the pancreas and the adrenal medulla in which the process is similarly dependent on the presence of calcium ions [see (102, 181)]. Thus, the extrusion of protein from the leucocidin-treated cell may resemble that of a natural secretion mechanism. It is distinct from the release of protein from the granules into the cytoplasm following the action of streptolysins O and S as observed by Hirsch, Bernheimer & Weissman [(60); see also Woodin (176)]. Hokin & Hokin showed that the secretion of protein induced in the pancreas was associated with increased incorporation of P^{32} in phospholipids, mainly in the phosphoinositide fraction (61). Woodin & Wieneke (178, 180) also showed that there was an increased incorporation of P^{32} in the phospholipid of leucocidin-treated leucocytes, mainly in an acid lipid fraction that was not phosphoinositide. However, they showed a stimulation of incorporation of P^{32} into nucleotides and other acid-soluble compounds as well. There appeared to be no causal connection between the stimulated incorporation of P^{32} into these compounds and the extrusion of protein by the leucocidin-treated cell. Antileucocidin failed to inhibit the incorporation of P^{32} in leucocidin-treated cells, which suggested that the stimulated P^{32} incorporation was a consequence rather than a concomitant of the action of the leucocidin. The stimulated P^{32} incorporation probably resulted not from an increasing rate of turnover of phosphorus compounds in the cell, but from the direct utilization of external orthophosphate at the surface of the cell (180).

The immediate mode of action of leucocidin is still obscure. In the polymorphonuclear leucocyte it brings about some change that causes the granules to lose their streaming motion and to go into Brownian movement. The changes that take place after that, the fusion of the granules to the cell walls and the discharge of their contents through the cell wall, seem to be independent of the leucocidin and dependent on the presence of calcium and ATP. The extrusion of granular protein is a secondary consequence of the action of the leucocidin. Indeed, granules do not appear to be necessarily involved in the action of leucocidin since macrophages are just as susceptible as polymorphonuclear leucocytes to the action of leucocidin and yet they do not contain granules, and very little protein is released from them on treatment with leucocidin. Both leucocytes and macrophages adsorb the leucocidin onto their surfaces and undergo a loss in glycolytic activity and a change in permeability to potassium ions. There is an accumulation of inorganic phosphorus and a decrease in ATP which is not due to acid phosphatase action. It is possible that the leucocidin adsorbed on the cell surface sets up a chain of reactions, one stage of which requires ATP (176). Since the perme-

ability changes in the surface of the leucocyte and the macrophage are extremely restricted (i.e., to cations and water), Woodin & Wieneke (182) suggested that the primary cytotoxic effect of leucocidin is to cause a membrane depolarization.

The leucocidin preparation used by Woodin is most interesting. It consists of two components that act synergistically but are inactive singly. In all the work discussed above, a mixture of the two components was used. Woodin showed that the yield of leucocidin from cultures of the staphylococcus grown on CCY medium (47) could be increased tenfold by substituting a diffusate of Oxoid yeast for a diffusate of Bacto yeast (174). He found that when a partially purified preparation of the leucocidin was processed on Amberlite CG-50 or carboxymethylcellulose columns, a single trailing protein peak emerged which was not coincident with the leucocidin peak. The leucocidin peak accounted for only a small proportion of the total leucocidin placed on the column, but when all the fractions taken from the column were united the recovery of leucocidin was complete. This suggested that more than one substance was necessary for leucocidin reaction. He then separated two kinetically and immunologically homogeneous crystalline proteins by fractionation on Dowex-2 and Amberlite CG-50 columns, called F and S according to whether they eluted form the columns fast or slowly. Their molecular weights were 32,000 and 38,000, respectively (175). It is not known whether these components interact, or how they act together. They are both adsorbed to the leucocyte from solutions containing either or both of the components (175). In the assay of leucocidin, and of antileucocidin, it is obviously necessary to assay each of these components and their homologous antibodies separately, taking into account the presence of the other. Such methods of assay have been described (46, 175, 177).

Staphylococcal enterotoxin.—Bergdoll, Sugiyama & Dack are to be congratulated for their dauntless effort and achievement in obtaining the staphylococcal enterotoxin in a high state of purity (13). This toxin is a common cause of food poisoning and its relation to disease is probably more clearly established than that of any other staphylococcal toxin. It is therefore a toxin of great medical interest, but research on it has hitherto been almost impossible because of the difficulty of assay. Of all the species of animal that have been used for the detection of the enterotoxin, only the rhesus monkey and man have proved to be reliable. The test is made by feeding the sample of toxin in solution to young monkeys by catheter; the animals are observed for 5 hr and vomiting is taken as a positive reaction. Since monkeys rapidly develop a resistance to the toxin, new animals should always be used. To obtain a roughly quantitative assay on a single sample of toxin it is necessary to administer several fivefold dilutions each to six monkeys (13, 146).

The toxin was purified in five steps: acid precipitation, alumina adsorption, alcohol precipitation, Amberlite IRC-50 adsorption, and starch electrophoresis. The final product was homogeneous by simple ultracentrifugal and

electrophoretic criteria. It had a mixed average molecular weight of 24,000. The amino acid composition was determined and carbohydrate and lipid were shown to be absent (57). The purified toxin was antigenic and immunologically homogeneous. It was therefore possible to prepare monovalent sera which have proved to be useful in assaying the toxin *in vitro* by means of a gel diffusion technique (14, 135), and in the selection of enterotoxin-producing strains of the staphylococcus (145).

Purified preparations of staphylococcal enterotoxin increase the dermal reactivity of rabbit skin to adrenaline. This effect is not neutralizable with antitoxin. Whether it is due to the enterotoxin is not yet known (20).

The Neurotoxins

Dysentery toxin.—The most powerful toxins are tetanus and botulinum toxins and the simple protein toxin (as distinct from the endotoxin) of *Shigella dysenteriae*. They all contain about a million LD_{50}/mg per kg for susceptible animals, and they all produce neurological symptoms. Dysentery toxin, however, is not a true neurotoxin; the neurological effects it produces are secondary to its action on blood vessels. The toxin was isolated in a kinetically and immunologically homogeneous condition by van Heyningen & Gladstone (160). Like diphtheria toxin, its production is inhibited in the presence of an excess of iron, but in this case toxin is not produced by the organism at the expense of cytochrome; on the contrary, as long as all the iron in the medium is being incorporated in bacterial cytochromes toxin production is optimal, and only when there is an excess of iron over that required for cytochrome production is toxin production inhibited (154).

The rabbit is the most susceptible species and in this animal the toxin produces typical neurological symptoms of flaccid paralysis, but Howard (62) and Bridgewater et al. (25), using purified toxin (160), have shown that in other species of animals (e.g., rats, hamsters) no neurological symptoms are produced. In these animals, the blood vessels of various organs are affected. In the rabbit, the affected blood vessels appear to be confined to the central nervous system, particularly the cervical enlargement of the spinal cord. As a result of this vascular damage there is a secondary destruction of motor nerve cells, but the neurological symptoms are probably due to the pressure resulting from edema in the spinal cord.

Botulinum toxin.—Work on botulinum and tetanus toxins to 1955 has been reviewed by Wright (183). Since that time some interesting observations have been made on the nature of botulinum toxin. Like several other bacterial toxins and enzymes, the toxins of at least three of the *Clostridium botulinum* types are produced as inactive protoxins which are convertible to active toxins by the action of proteolytic enzymes. Duff, Wright & Yarinsky (35) showed that the intraperitoneal and subcutaneous, but not the oral, toxicity of the type E toxin was greatly enhanced by treatment with trypsin. This was confirmed by Sakaguchi & Sakaguchi (131). Bonventre & Kempe

(21, 22, 23) showed that types A and B toxins were also produced as protoxins. *Clostridium botulinum* types A, B, and E have been shown to produce trypsin inhibitors (63). It is possible that these may play a part in the production of active toxin under natural conditions.

The size of botulinum toxin molecules seems to vary from particles of molecular weight about 70,000 to about 1,000,000. Wagman (164, 166) showed that the type A molecule, with a molecular weight of about 1,000,000, would dissociate under certain conditions of pH and ionic strength into particles with a molecular weight of about 70,000, some of which were atoxic and some more toxic than the original preparation. Heckley and colleagues (56, 58) showed that when botulinum type A toxin with a sedimentation coefficient of 17.9 was ingested by rats, particles with a sedimentation coefficient of 7.9 appeared in the lymph. There are suggestions of even smaller fragments of botulinum toxin. Gerwing, Dólman & Arnott (44, 45) purified the type E toxin and its protoxin. The protoxin had a sedimentation coefficient of 5.6, but the trypsin-activated toxin consisted of fragments which would not come away from the meniscus in an ultracentrifugal field of 260,000 $\times g$. They also found that some of the toxicity of the preparation would diffuse through dialyzing membranes. This partially purified, trypsin-activated preparation was less toxic (10 million MLD/mg N) and of lower molecular weight than a partially purified preparation of type E trypsin-activated toxin of Fiock, Yarinsky & Duff (38) which contained 45 million MLD/mgN and had a sedimentation coefficient of 4.7.

Wagman (165) showed that although type A toxin (sedimentation constant = 15.5) as isolated was insusceptible to the action of pepsin, it became susceptible when dissociated at pH 9.2 (sedimentation constant = 7). When the dissociated product was treated with pepsin, some of the fragments passed through dialyzing membranes and retained their toxicity. The digestion with pepsin was not controllable and therefore the results were variable. The dissociated product contained less than half as much tyrosine as the original material, and the toxic peptic digestion products that passed through dialyzing membranes contained no tyrosine at all. It was therefore suggested that these toxic fragments were originally located in those parts of the molecule between the free amino acid ends of the chain and the tyrosine residues nearest to these N-termini.

It is now clearly established that botulinum toxin acts on the peripheral nervous system by suppressing the output of acetylcholine from the endings of cholinergic motor nerves at the neuromuscular junction [see (183)]. Brooks (26) showed that the toxin acted at the tips of the nerve endings where it caused a reduction of the frequency of miniature end plate potentials without changing their mean amplitude. This view was supported by Hughes & Whaler (64) who showed that factors that influence the release or metabolism of acetylcholine (e.g., increased nerve ending activity, anticholinesterase drugs, atropine) increased or decreased the rate of paralysis by the toxin

according to whether they increased or decreased the depolarization phase in the prejunctional region. They suggested that the toxin might enter the prejunctional membrane while its permeability was increased during depolarization, perhaps through the gaps through which the acetylcholine quanta were released.

Katz [quoted by Thesleff (149)] showed that the electron microscopic appearance of the botulinum-intoxicated neuromuscular junction was quite normal; there was no change in the number or size of the vesicles which are thought to contain the acetylcholine. In mammalian skeletal muscle intoxicated with botulinum toxin, the entire muscle membrane became susceptible to applied acetylcholine in the same way as a chronically denervated muscle (149).

Tyler (151) observed an instance of the action of botulinum toxin on the central nervous system. Electrical stimulation of multiple peripheral nerves in a human patient with botulism elicited "H" reflexes that were suggestive of central action. This could have been due to a direct inhibitory action on acetylcholine-stimulated inhibitory interneurones of the cord, such as Renshaw cells.

Tetanus toxin.—Kryzhanovskii (73, 74, 75) has taken up the old problem of the spread of tetanus toxin within the body [see Wright (183)] and has gained further support for the view that in local tetanus the toxin travels up the regional motor nerve trunks to the anterior horns of the spinal cord. He criticizes early experiments on the interruption of the sensory inervation of toxin-injected muscles on the grounds that trauma and interference with the blood supply obscured the results, and that the experiments were generally done on single animals. Kryzhanovskii divided the sensory and motor roots of large numbers of rats subdurally in such a way as to avoid trauma and to enable the animals to survive well with no locomotor disturbances. Toxin was injected into the muscles of these animals while antitoxin was administered intravenously to neutralize systemic toxin. Tetanus developed in animals in which the sensory roots were divided, and failed completely to develop in animals whose motor roots were divided. Measurements of toxin were made in portions of nerve, again in large numbers of intramuscularly injected rats. Toxin was observed in the sciatic and femoral nerves and the anterior roots on the ipsilateral side, but not in the posterior roots or in the nerves and roots on the contralateral side. The epineuria and their lymphatic pathways were not involved in the transport of toxin along nerve trunks since the roots are devoid of these, and yet toxin was found in them. Similar results were obtained with large numbers of other small animals (mice, guinea pigs, rabbits, cats) and with donkeys.

There is no doubt that tetanus toxin acts upon the central nervous system. Brooks, Curtis & Eccles (27) showed that it acts, like strychnine, by suppressing synaptic inhibition. It is not known whether it acts pre- or postsynaptically at the inhibitory synapses, or whether it in any way affects the as yet unidentified inhibitory transmitter. Although the main action of teta-

nus toxin is central, there are several suggestions that it may have a peripheral influence as well. Such peripheral action would generally be masked by the central effects. Ambache and his colleagues observed that both botulinum and tetanus toxins paralyzed the cholinergic sphincter pupillae of the eye. Both toxins had the same action, and in the case of tetanus toxin it was shown that there was a reduction in the output of acetylcholine by the nerve endings (2, 3, 4). They pointed out that there were minor clinical features of tetanus in man which suggested that other parasympathetic motor nerves may be affected. These included retention of urine and atonicity of the bladder, tachycardia, and constipation (4). We will return to this point.

In seeking a biochemical means of approaching the problem of the mode of action of tetanus toxin, van Heyningen reinvestigated the specific fixation of the toxin by the grey matter of nervous tissue [the Wasserman-Takaki phenomenon (17, 155–159, 162, 163)]. He showed that the substance responsible for this fixation was a ganglioside. The fixation was toxin-specific and ganglioside-specific. No other toxin or protein tested, except tetanus toxoid, was fixed by ganglioside in a comparable way. At high concentrations of both reactants, ganglioside fixed up to 20 times its weight of toxin. Toxoid was fixed much less avidly. The sialic acid and hexosamine residues of ganglioside were essential for fixation, but a Tay-Sachs ganglioside containing both these residues did not fix toxin. Ganglioside is water-soluble, but forms water-insoluble complexes (mixed micelles?) with water-insoluble cerebrosides and sphingomyelin. At low concentrations of toxin, the fixing capacity of ganglioside was considerably increased when it was complexed with a particular proportion of cerebroside, although cerebroside alone does not fix toxin. Thus, complexes of cerebroside-ganglioside containing 25 percent ganglioside fixed up to 200 times as much toxin as complexes containing 5 or 50 percent ganglioside (161). This suggested possibilities for specialized toxin-receptor patches in nervous tissue. In this connexion it is interesting to note that the brain of the relatively toxin-resistant frog has a very low toxin-fixing capacity although its ganglioside content does not differ markedly from that of the brain of other species. But whereas in other species, the brain ganglioside cannot be extracted with water (presumably because the ganglioside is complexed with cerebrosides and other water-insoluble substances), in the frog brain the ganglioside is water-soluble (163). This suggests that the frog brain ganglioside is not complexed with cerebroside (or other substances), which may account for the low toxin-fixing capacity of the brains of these animals, and possibly for their low susceptibility to tetanus toxin.

Ganglioside did not appear to be changed in any way by tetanus toxin, and its role, if any, in the action of the toxin is not yet known. There is some evidence, which can by no means be regarded as conclusive, to suggest that fixation of toxin by nervous tissue is essential for the lethal action of the toxin. Wolters & Fischoeder (173) and Fulthorpe (41) observed that prior treatment of brain tissue with toxoid reduced its capacity to fix toxin. Several workers [see Wright (183)] have shown that prior injection of large doses of

toxoid into various species of animal protected these animals against otherwise lethal doses of toxin injected a few hours to a few days later, before the formation of specific antibody. It could be argued that, by preventing the fixation of toxin by nervous tissue (i.e., by ganglioside), the toxoid prevents the lethal action of the toxin, and therefore that ganglioside plays a part in the action of the toxin. But certain other toxoids protect against their homologous toxins in a similar manner (96), and it may be that this is a more general phenomenon, not specifically concerned with tetanus.

We have mentioned the possible peripheral effects of tetanus toxin, and the similarity of its action to that of botulinum toxin in the eye. Both of these neurotoxins are derived from closely related species of organism and it is possible they have fundamentally the same mode of action. If this is the case, then ganglioside would not be concerned, since botulinum toxin is not fixed by it. The fixation of tetanus toxin by ganglioside may be responsible for diverting it to the central nervous system and throwing its peripheral action into the background. Davies & Wright (32) noticed that animals whose death from the effects of tetanus was delayed by the administration of toxoid did not show the usual spastic signs of tetanus, but showed flaccid paralytic signs similar to those of botulinum intoxication.

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